

## Changes in Spores of *Bacillus megaterium* Treated with Thioglycolate at a Low pH and Restoration of Germinability and Heat Resistance by Cations<sup>1</sup>

DURWOOD B. ROWLEY AND HILLEL S. LEVINSON

Food Division and Pioneering Research Division, U. S. Army Natick Laboratories, Natick, Massachusetts

Received for publication 14 November 1966

Spores of *Bacillus megaterium* QM B1551 treated with thioglycolate (0.4 M, pH 2.6) at 50 C for 30 min remained refractile, but they became stainable, lysozyme-sensitive, and nonviable, and they lost dipicolinic acid (DPA). The loss of DPA and of viability were functions of the time and temperature of exposure to thioglycolate. Spores treated with thioglycolate at a lower temperature and for a shorter time (30 C, 5 min) retained DPA, viability, and nonstainability. Although these spores also retained their resistance to  $\gamma$  radiation and to lysozyme, they lost thermo-resistance. Their percentage of germination over a 2-hr period in glucose was markedly reduced. Germinability and heat resistance were restored by exogenous cations, suggesting that the thioglycolate treatment (30 C, 5 min) resulted in the loss of spore ions essential for normal germination in glucose and for heat resistance.

The chemical composition of the spore plays an important role in its response to the environment. Certain spore properties may be modified, without loss of viability, through alteration of the spore's chemical make-up by treatment at a low pH, with (3) or without (1, 7, 8) a reducing agent. Gould and Hitchins (3) demonstrated that spores treated with thioglycolate at pH 3.0 were rendered sensitive to lysozyme, and suggested that the reducing agent exposed lysozyme-sensitive mucopeptide by rupturing spore coat disulfide bonds. Other investigators have concluded that, when spores were exposed to an acid environment for 1 to 3 hr, there was an exchange of ions between the spore and its surrounding environment, affecting dormancy (7), heat resistance (1), and germinability (8).

In this paper, we report on the loss, as a result of exposure to thioglycolate at a low pH, of spore nonstainability, dipicolinic acid (DPA), viability, lysozyme resistance, heat stability, and capacity to germinate in glucose, as well as on the restoration by cations of the germinability and heat resistance of thioglycolate-treated spores.

### MATERIALS AND METHODS

*Spore preparation.* Spores of *Bacillus megaterium* (QM B1551) harvested from a medium containing

<sup>1</sup> Presented in part at the 66th Annual Meeting, American Society for Microbiology, Los Angeles, Calif., 1-5 May 1966.

0.5% Wilson's Liver Fraction "B" (6), buffered at pH 6.8 with 0.01 M potassium phosphate, were washed by repeated centrifugation at 4 C, lyophilized, and stored over CaSO<sub>4</sub> at 4 C until used. Fewer than 1% of these refractile (phase-bright), lysozyme-resistant spores (containing 137  $\mu$ g of DPA and 50  $\mu$ g of calcium per mg) were stainable with 0.5% methylene blue.

*Treatment of spores with thioglycolate.* Thioglycolate was prepared just before use by adjusting thioglycolic acid (99.6%; Fisher Scientific Co., Pittsburgh, Pa.) to the desired pH with 5.5 N NaOH and diluting to the appropriate concentration with a buffer. Spores (2.5 mg/ml) were treated (i) with thioglycolate (thioglycolate-treated), (ii) with glass-distilled water (water-treated), or (iii) with buffer at the temperatures, pH levels, and concentrations described with individual experiments. Spores which were treated with glycine-HCl buffer (pH 2.6) are referred to as acid-treated spores. After treatment, thoroughly washed spores were examined for stainability, loss of refractility (phase darkening), lysozyme sensitivity, heat or radiation resistance, and for germination in glucose.

*Estimation of changes in treated spore suspensions.* Turbidity of spore suspensions (0.5 mg of spores per ml of thioglycolate) was measured in a Klett-Summers colorimeter with a no. 56 (530 to 590 m $\mu$ ) filter. Lysozyme sensitization was indicated by darkening (under phase-contrast) of spores (2.5 mg/ml) which had been incubated for 15 min at 37 C with lysozyme (1 mg/ml) in 0.067 M phosphate buffer, pH 8 (3). DPA in the supernatant liquid of treated spores was estimated colorimetrically (4).

For studies on germination, unless otherwise

specified, aqueous suspensions of spores (2.5 mg/ml) were heat-activated (60 C, 15 min) and diluted with an equal volume of water. To 50-ml Erlenmeyer flasks, containing 0.6 ml of the germination medium, were added 2.4 ml of the diluted spore suspension (1.25 mg/ml) to give concentrations of substrate as indicated in the individual experiments, and 1.0 mg of spores (ca.  $3 \times 10^8$  spores) per ml. Germination (per cent staining with 0.5% aqueous methylene blue) was determined after 2 hr of incubation at 30 C on a reciprocal shaker.

Viable spores were those capable of forming colonies after incubation at 30 C for 24 hr on Nutrient Agar (Difco) plus 0.1% yeast extract. During thioglycolate treatment, 1.0-ml samples were removed, immediately diluted with 0.05 M potassium phosphate (pH 7.0), and plated. Heat and radiation resistance were determined as described in the individual experiments. The 1.0 Mc, 4.2 Mrad per hr,  $^{60}\text{Co}$  facility at the U.S. Army Natick Laboratories, Natick, Mass., was the  $\gamma$ -radiation source.

### RESULTS

*Changes induced by thioglycolate.* Certain germination-like changes, such as lysozyme sensitization (2), reduction in turbidity of spore suspensions, release of DPA, and acquisition of stainability, occurred when spores, suspended in thioglycolate, buffered with glycine-HCl (pH 2.6), were incubated at 50 C. Lysozyme sensitization of *B. megaterium* spores by exposure to thioglycolate at 50 C increased with increasing  $\text{H}^+$  (Fig. 1), with increasing thioglycolate concen-

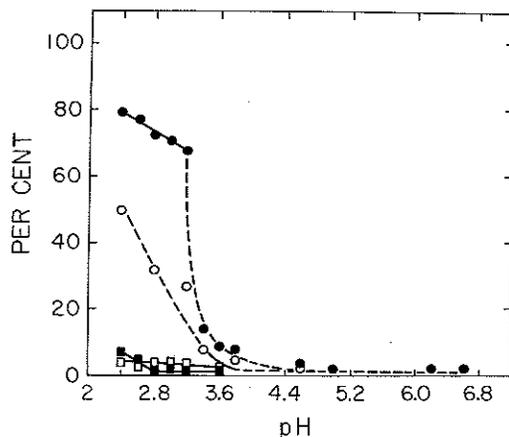


FIG. 1. Effect of pH on thioglycolate-produced stainability and lysozyme sensitivity of *Bacillus megaterium* spores. Spores were treated at 50 C for 2 hr with buffered 0.1 M thioglycolate (circles) or with 0.05 M buffers (squares). Glycine-HCl buffer was used for pH 2.4 to 3.6; citrate-phosphate, for pH 3.8 to 6.6. Treated spores, washed three times with distilled water and resuspended in 0.067 M phosphate buffer (pH 8.0), were examined for stainability (solid symbols) and for lysozyme sensitivity (open symbols).

tration (Fig. 2), and with time of exposure (Table 1). Further evidence for a change in spore structure, as postulated by Gould and Hitchins (3), was the increased stainability of the treated spores (Fig. 1, Table 1). After a 30-min treatment, during which viability decreased markedly, the turbidity of spore suspensions had decreased (22%), 63% of the spores had become stainable, and 66% of the DPA had been released, but the spores remained refractile (Table 1). Similar treatment (50 C) with glycine-HCl buffer (pH 2.6), without thioglycolate, had no effect on spore viability. Release of DPA and loss of viability decreased with decreasing temperature

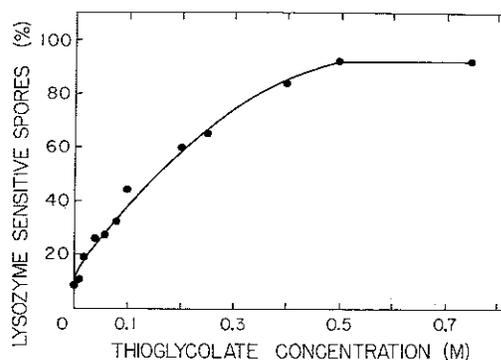


FIG. 2. Effect of thioglycolate concentration on lysozyme sensitization of *Bacillus megaterium* spores. Spores, treated with thioglycolate (pH 2.6) at 50 C for 2 hr, were washed, resuspended (as in Fig. 1), and tested for lysozyme sensitivity.

TABLE 1. Changes induced by thioglycolate in spores of *Bacillus megaterium*\*

Time at 50 C	Turbidity loss	DPA loss	Stainable spores	Lysozyme-sensitive spores	Nonviable spores
min	%	%	%	%	%
0	0	0	<1	<1	0
10	7	25	30	29	25
20	15	51	42	37	59
30	22	66	63	44	90
Control (30 min)	5	6	9	4	0

\* Spores were treated at 50 C for the indicated times with 0.4 M thioglycolate (buffered with glycine-HCl, 0.05 M, pH 2.6) or with the pH 2.6 buffer alone (control). Turbidity is indicated as per cent loss of original optical density. Dipicolinic acid (DPA) loss is indicated as per cent of total spore DPA. Treated spores (washed and resuspended as in Fig. 1) were examined for refractility under phase-contrast optics (no change), for stainability, and for lysozyme sensitivity.

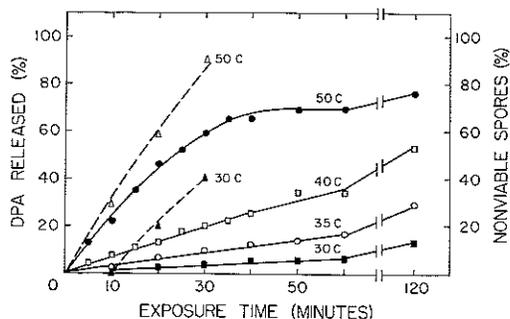


FIG. 3. Effect of time and temperature of thioglycolate (0.4 M, pH 2.6) treatment on *Bacillus megaterium* spore dipicolinic acid (DPA) release and viability. DPA (solid lines) and viability loss (dashed lines) are represented, respectively, as per cent of total spore DPA which was released and as per cent of spores which became nonviable during thioglycolate treatment. DPA values were corrected for acid-treated controls (without thioglycolate) which lost no viability.

and time of exposure to thioglycolate (Fig. 3). There was no appreciable loss of DPA, viability, or lysozyme resistance during a 10-min thioglycolate treatment at 30 C. It may be noted that, using different experimental conditions (thioglycolic acid, 1%, v/v; urea, 8 M; 37 C; 90 min), Gould and Hitchins (3) showed that *B. cereus* spores became lysozyme-sensitive without loss in viability.

*Germination of thioglycolate-treated spores.* Spores treated with thioglycolate (0.4 M, pH 2.6) at 30 C for a short time (5 min) remained viable and retained refractility, nonstainability, lysozyme resistance, and DPA. Yet, this treatment markedly reduced the germination of spores in glucose (Table 2). However, germinability was regained when calcium salts (acetate, nitrate, or chloride) were added to the glucose germination medium. A similar low-temperature acid treatment of spores, without thioglycolate, had little effect on germinability, but by extension of the time of acid treatment to 2 hr (Table 2) the spores were modified in the same way (reduced spore germinability in glucose) as by short-time exposure to thioglycolate. These acid-treated spores also responded to the addition of calcium. Thioglycolate may have enhanced the loss of spore cations at acid pH (1), removing those essential to germination in glucose.

Exchangeable spore calcium is important for germination of *B. megaterium* strain Texas in an alanine-inosine mixture (8). The calcium accumulating during sporogenesis may play a significant role in the glucose-induced germination of *B. megaterium* spores (5). If thioglycolate treatment destroyed the ability of spores to germinate on glucose through loss of cations essential for germination, replacing these cations in the spore should restore germinability. Indeed, exposure to calcium, followed by thorough wash-

TABLE 2. Effect of certain salts in restoring the germinability of *Bacillus megaterium* spores previously treated with thioglycolate<sup>a</sup>

Germination medium	Germination <sup>b</sup> of spores treated with					
	No treatment	Distilled water		Glycine-HCl (pH 2.6)		Thioglycolate, 5 min
		5 min	2 hr	5 min	2 hr	
Glucose	90	90	80	82	6	9
+ potassium acetate	93	96	90	72	20	6
+ calcium acetate	92	95	93	95	80	94
+ calcium nitrate		91		84		88
+ calcium chloride		88		90		75
+ sodium acetate		85		82		15
+ cobalt acetate		80		78		5
+ magnesium acetate		90				50
+ manganous acetate		93		95		91
+ barium acetate		89				76

<sup>a</sup> Spores were treated (30 C, 5 min) with distilled water; with glycine-HCl (0.05 M, pH 2.6); or with glycine-HCl buffered thioglycolate (0.4 M, pH 2.6). Water and glycine-HCl treatments were also extended to 2 hr. Treated spores were thoroughly washed with distilled water. Washed spores were heat-activated (60 C, 15 min) in a water suspension, cooled, diluted, and added to the germination medium: D-glucose, 0.01 M; salts, 0.02 M.

<sup>b</sup> Percentage of spores stainable with 0.5% methylene blue after 2 hr in germination medium at 30 C. Neither treated nor untreated spores germinated in the salts without glucose.

TABLE 3. Restoration by calcium of the glucose germinability of thioglycolate-treated spores of *Bacillus megaterium*<sup>a</sup>

Post-thioglycolate exposure		Germination %
Initial	Final	
Not heat-activated		
Water, 30 C, 30 min	None	0
Ca <sup>++</sup> , 30 C, 30 min	None	30
Heat-activated		
Water, 30 C, 30 min	Water, 60 C, 15 min	1
Ca <sup>++</sup> , 30 C, 30 min	Water, 60 C, 15 min	>90
Ca <sup>++</sup> , 60 C, 15 min	Water, 30 C, 30 min	>90
Water, 60 C, 15 min	Ca <sup>++</sup> , 30 C, 30 min	>90

<sup>a</sup> Spores, treated with thioglycolate and washed as indicated in Table 2, were suspended in water or in calcium acetate (0.02 M), subjected to the indicated post-thioglycolate treatments, washed thoroughly after the initial and after the final post-thioglycolate treatments, and germinated in 0.01 M D-glucose. Glucose-induced germination of spores which had not been treated with thioglycolate: 30% without heat activation, 90% with heat activation.

ing, restored the ability of thioglycolate-treated spores to germinate in glucose (Table 3). Calcium was equally effective in restoring the germinability of heat-activated spores, whether an exogenous source was present prior to, during, or after the heat treatment. As little as 1.5 mM calcium acetate, whether present during heat activation or together with glucose in the germination medium, enabled more than 80% of the thioglycolate-treated spores to germinate (Fig. 4). Other divalent cations (Mg<sup>++</sup>, Mn<sup>++</sup>, or Ba<sup>++</sup>) were effective in replacing calcium in the germination medium, but the monovalent cations and one of the divalent cations (Co<sup>++</sup>) had no effect (Table 2). Apparently, heat-activated thioglycolate-treated spores required an exogenously supplied divalent cation, such as calcium, for maximal germination.

**Heat resistance.** Spores exposed to thioglycolate (0.4 M, pH 2.6), under conditions (30 C, 5 min) too mild to induce lysozyme sensitivity, became heat-sensitive (Fig. 5). Neither acid treatment for 5 min, without thioglycolate (Fig. 5), nor exposure to pH 6.0 thioglycolate (data not shown) affected the heat resistance of the spores. As with germinability, exposure to Ca<sup>++</sup> reversed the effect of thioglycolate; i.e., the spores reverted to heat resistance. Survival curves for water- or acid (glycine-HCl)-treated spores, which were heat-challenged after exposure to Ca<sup>++</sup> (data not shown), were virtually superimposable on the curve for thioglycolate-treated spores similarly exposed to Ca<sup>++</sup> (Fig. 5).

**Radiation resistance.** The low temperature-short time thioglycolate treatment had no effect on spore resistance to  $\gamma$ -radiation (Fig. 6). We have suggested that the thioglycolate-induced

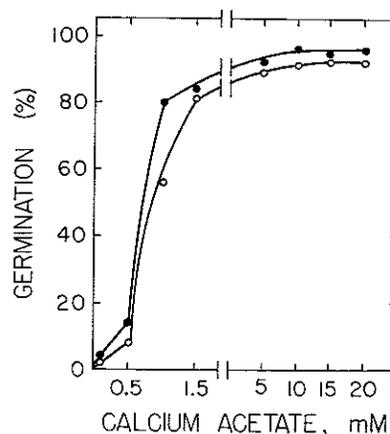


FIG. 4. Effect of calcium concentration on the restoration of the glucose germinability of thioglycolate-treated *Bacillus megaterium* spores. Spores were treated (30 C, 5 min) with thioglycolate (0.4 M, pH 2.6) buffered with glycine-HCl, and either were heat-activated (60 C, 15 min) in water, washed thoroughly, and germinated in 0.01 M glucose plus graded concentrations of calcium acetate (O), or were heated in graded concentrations of calcium acetate, washed thoroughly, and germinated in 0.01 M glucose (●).

reduction of germinability in glucose (Table 2) and reduction of heat resistance (Fig. 5) were due to the loss of cations. Since other investigators have indicated that various spore ions have no apparent role in spore resistance to  $\gamma$ -radiation (11) or to ultraviolet irradiation (10), it was therefore not surprising that the mild treatment with thioglycolate had no effect on this facet of spore behavior.

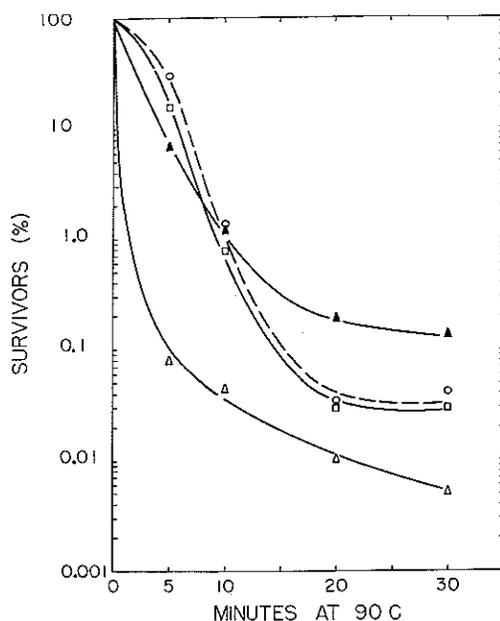


FIG. 5. Heat resistance of thioglycolate-treated *Bacillus megaterium* spores before and after treatment with calcium. Spores, treated (30 C, 5 min) with 0.4 M thioglycolate buffered with pH 2.6 glycine-HCl ( $\Delta$ ), with 0.05 M, pH 2.6 glycine-HCl in the absence of thioglycolate ( $\square$ ), or with distilled water ( $\circ$ ), were washed thoroughly and suspended in water. Another portion of washed thioglycolate-treated spores was suspended in 0.02 M calcium acetate ( $\blacktriangle$ ). The aqueous suspensions and the calcium acetate suspension were incubated at 30 C for 30 min. Spores were again thoroughly washed, and aqueous suspensions of spores (2.5 mg/ml) were heated at 90 C for the times indicated. Appropriate dilutions of the heated spores were plated on Nutrient Agar (Difco) plus 0.1% yeast extract.

#### DISCUSSION

It has previously been reported that exposure of spores to a low pH results in the loss of spore cations (5, 9), and that reversible changes in heat resistance (1) and in germinability (8) depend upon calcium and other cations. We suggest the loss of ions from spores, treated with thioglycolate at low pH, as the basis for the reduction in both heat resistance and germinability. No exclusive role in this regard is claimed for  $\text{Ca}^{++}$ . Indeed, we have determined that several other divalent cations are capable of restoring germinability in glucose. Gould and Hitchins (3) postulated that spore coat disulfide bonds may be unmasked or may be more reactive at low pH, or both, with consequent increased susceptibility to rupture by thioglycolate. If this were the case, by rupturing such bonds, thioglycolate

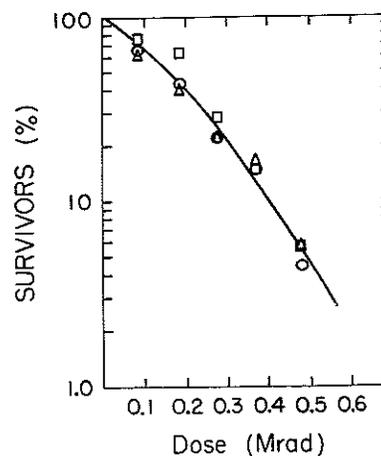


FIG. 6. Effect of thioglycolate treatment on the resistance of *Bacillus megaterium* spores to  $\gamma$ -radiation. Spores, treated (30 C, 5 min) with thioglycolate (0.4 M, pH 2.6) buffered with glycine-HCl ( $\Delta$ ), with 0.05 M, pH 2.6 glycine-HCl ( $\square$ ), or with distilled water ( $\circ$ ), were washed thoroughly and suspended in water. After irradiation at 0 C in a  $^{60}\text{Co}$  source, the aqueous suspensions were diluted with water and plated.

might enhance an exchange of spore cations such as has been reported (1) to occur at acid pH. The physiological evidence on germination in glucose, presented in this paper, did suggest that the effect of thioglycolate at low pH was duplicated by exposure of spores for more extended periods at low pH in the absence of thioglycolate. However, in view of the lack of direct evidence that thioglycolate at low pH is effective in disrupting disulfide bonds of polymers in general, or of bacterial spores in particular, caution should be exercised in postulating this mechanism for the role of thioglycolate in enhancing the alteration of spore properties.

#### ACKNOWLEDGMENTS

We thank Muriel Stern and Ronald Joseph for technical assistance, and H. M. El-Bisi, G. R. Mandels, and E. T. Reese for critical reviews of the manuscript.

#### LITERATURE CITED

- ALDERTON, G., AND N. S. SNELL. 1963. Base exchange and heat resistance in bacterial spores. *Biochem. Biophys. Res. Commun.* **10**:139-143.
- DEMAIN, A. L., AND J. F. NEWKIRK. 1960. Dissociation of spore germination from outgrowth by use of auxotrophic mutants of *Bacillus subtilis*. *J. Bacteriol.* **79**:783-788.
- GOULD, G. W., AND A. D. HITCHINS. 1963. Sensitization of bacterial spores to lysozyme

- and to hydrogen peroxide with agents which rupture disulphide bonds. *J. Gen. Microbiol.* **33**:413-423.
4. JANSSEN, F. W., A. J. LUND, AND L. E. ANDERSON. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**:26-27.
  5. LEVINSON, H. S., AND M. T. HYATT. 1964. Effect of sporulation medium on heat resistance, chemical composition, and germination of *Bacillus megaterium* spores. *J. Bacteriol.* **87**:876-886.
  6. LEVINSON, H. S., AND M. G. SEVAG. 1953. Stimulation of germination and respiration of the spores of *Bacillus megaterium* by manganese and monovalent anions. *J. Gen. Physiol.* **36**:617-629.
  7. LEWIS, J. C., N. S. SNELL, AND G. ALDERTON. 1965. Dormancy and activation of bacterial spores, p. 47-54. *In* L. L. Campbell and H. O. Halvorson [ed.], *Spores III*. American Society for Microbiology, Ann Arbor, Mich.
  8. RODE, L. J., AND J. W. FOSTER. 1966. Influence of exchangeable ions on germinability of bacterial spores. *J. Bacteriol.* **91**:1582-1588.
  9. RODE, L. J., AND J. W. FOSTER. 1966. Quantitative aspects of exchangeable calcium in spores of *Bacillus megaterium*. *J. Bacteriol.* **91**:1589-1593.
  10. SLEPECKY, R., AND J. W. FOSTER. 1959. Alterations in metal content of spores of *Bacillus megaterium* and the effect on some spore properties. *J. Bacteriol.* **78**:117-123.
  11. TALLENTIRE, A., AND C. O. CHIORI. 1963. Heat and gamma-radiation resistance of *Bacillus megaterium* spores. *J. Pharm. Pharmacol. Suppl.* **15**:148T-149T.